β-MANNANASES OF FUNGI

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Abstract

β-1,4-Mannanases are of common occurrence in fungi. They are induced enzymes, the yields of enzyme being 10- to 100-fold greater when the organism is grown on mannan. All of the β-mannanases studied are endopolysaccharases acting on long chains in a random manner and unable to act on mannotriose or mannobiose. Acting on substituted mannans, they yield products containing the branch. Most of the microbial mannanase preparations contain glycosidases capable of removing galactose or glucose units from the mannan chain. The few which lack glycosidases are useful in the production of oligosaccharide products.

Introduction

The β-mannanase (E.C.3.2.1. . β-1,4-mannan 4-mannanohydrolase) activity of enzyme preparations has been studied for a number of years. Biologists have reported organisms which actively produce this enzyme in fungi (7, 11, 18); bacteria (15, 16, 30); higher plants (6, 8, 9; locust, coffee, almond, apricot); and in animals (10, 12; mammals; snails, oysters). It has been found also as a contaminant in crude commercial enzyme preparations (1, 11). β-Mannanase and related enzymes have been used in determining the structure of glucomannans (5, 21, 27) and of galactomannans (6–12, 29) by Whistler (28, 29), Bishop (21), Deuel (13), Courtois (6–12), and others.

With the establishment of the structure of some of the mannans (2, 4, 19, 20, 24, 26, 27, 29), a reinvestigation of their enzymatic hydrolysis should lead to further elucidation of the action of the various enzyme components. How, for example, do branching and the presence of a second sugar in the polymer affect β-mannanase activity? Does the polysaccharase act before the de-branching enzyme to produce branched fragments? Which linkages are susceptible to hydrolysis? What is the nature of the de-branching enzyme? Knowledge of this sort is necessary if these enzymes are to be useful in structural studies of other polymers.

Our previous work (23) revolved about the hydrolysis of relatively simple homopolysaccharides (glucans, fructans, etc.). Now the use of more complex materials, for example, those with mixed linkages, mixed sugars, and branching, is leading to a better understanding of the action of the endopolysaccharases (22, 23). In turning to the mannans, we find a series of polymers of intermediate complexity. Hopefully, some day we may be able to apply our knowledge in a logical manner to the unravelling of more complicated structures.

Methods

A. Preparation of Substrates

The preparation of β-1,4-mannans, glucomannans, and galactomannans has been reviewed recently by Smith and Montgomery (24). Our preparations

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were made for use as substrates for growth of the organisms and as substrates for determination of enzyme activity. Ivory nut mannan was obtained from the alkaline extract of ivory nut meal by precipitation with acetic acid. Further purification was effected by use of Fehling's solution (24).

Ivory nut mannan is a linear homopolymer. Like cellulose, it is water-insoluble unless modified. Galactose side groups, or the interruption of the mannan backbone by glucose units, confers water solubility. The variation in viscosity of such solutions with degree of polymerization (DP) can be used to assay mannanase activity. Alternatively, reducing group methods may be used.

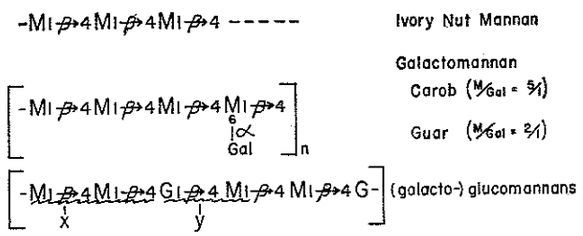

Fig. 1.

Carob gum (DP ca. 1500; Hui (14)) and guar gum (DP ca. 900; Hui (14)) were obtained from Stein Hall Co. They contained 17 and 34% galactose, respectively (galactose oxidase determination). Glucomannans were gifts from Drs. Timell and Nisizawa. O-β-D-Mannapyranosyl (1 → 4) D-mannose: mannobiose (M2) and O-β-D-mannopyranosyl (1 → 4) O-β-D-mannopyranosyl (1 → 4) D-mannose: mannotriose (M3) were prepared by enzymatic hydrolysis of mannan (see Table IV); the reduced dimer and trimer by treatment with borohydride.

Methyl α-D-galactopyranoside was obtained from Dr. Derek Ball; methyl β-D-mannopyranoside was prepared by the method of Bates *et al.* (3). Authentic samples of the following sugars, sufficient for use as markers on paper chromatograms, were supplied by Dr. Whistler and by Dr. Bishop: O-β-D-mannopyranosyl (1 → 4) D-mannose; O-β-D-mannopyranosyl (1 → 4) O-β-D-mannopyranosyl (1 → 4) mannose; O-α-D-galactopyranosyl (1 → 6) D-mannose; O-β-D-mannopyranosyl (1 → 4) D-glucose; O-β-D-glucopyranosyl (1→ 4) D-mannose.

B. Production of β-1,4-Mannanase

The organisms were grown on a basal medium containing (per liter): KH_2PO_4, 2.0 g; $(NH_4)_2SO_4$, 1.4 g; urea, 0.3 g; $MgSO_4.7H_2O$, 0.3 g; $CaCl_2$, 0.3 g, and trace elements (Fe, 1.0 mg; Mn, 0.5 mg; Co, 0.5 mg; Zn, 0.8 mg). Since the enzyme is adaptive, mannan (0.5%) was used as carbon source, either as ivory nut meal, ivory mannan, carob gum, or partially hydrolyzed carob gum. The addition of 0.01% yeast extract (Difco) and of 0.1% proteose peptone was usually found beneficial. The cultures were shaken on a reciprocal shaker at 29 °C for 2–3 weeks, during which period the filtrates were assayed for activity.

C. Estimation of Enzyme Activity

Enzyme solution (0.5 ml) was added to an equal volume of substrate (5 mg/ml in $M/20$ citrate buffer, pH 4.5) and incubated at 50° for 1 hour. Reducing sugars produced as a result of hydrolysis were determined by the dinitrosalicylic acid method (DNS) of Sumner and Somers (25). A solution has unit activity per milliliter if, when assayed as above, it produces 0.5 mg of reducing sugar as mannose.

The substrate selected for β-1,4-mannanase determinations was carob gum since it is water-soluble and rapidly hydrolyzed. Methyl β-mannoside and methyl α-galactoside were used as substrates for the determination of glycosidases. Both are somewhat resistant to hydrolysis and gave low unit values. A better indication of the activities of the glycosidases is the appearance of the simple sugars as hydrolysis products of the mannans, as seen on paper chromatograms.

D. Dry Enzyme Preparations

Cold culture filtrates of organisms were adjusted to pH 4.5 with $M/5$ citric acid, and 2 volumes of cold acetone were added. The precipitates formed were collected by filtration or centrifugation, washed with cold acetone, dried *in vacuo*, and stored in the cold.

TABLE I

Sources of β-1 → 4 mannanase

Source	QM No.	Dry enzyme preparations: Yield (max.),* units/ml	Dry enzyme preparations: β-1,4-mannanase, units/mg	Dry enzyme preparations: Type†
QM microbial cultures				
Aspergillus giganteus	7974	280	102	III
A. luchuensis	873	52	9	I
A. nidulans v. *echinulatus*	1909	40		II
Bacillus subtilis	B1564	125	60	IV
Epicoccum yuccae	284e	24		II (I)
Fusicoccum sp.	2045	150	16	
Memnoniella echinata	6859	20		II
Paecilomyces varioti	3408	265	100	II (I)
Penicillium expansum	6758	36		
P. funiculosum	474	84	98	I
P. isariiforme	1897	46	8	
P. ochro-chloron	477	41	30	I
P. piscarium	471	41		
P. verruculosum	7999	100	80	I (II)
P. wortmanni	1859	640	320	I
Trichurus spiralis	834	140	45	IV
Commercial sources				
Cellulase conc. 9 X (Takamine)			20	I
Hemicellulase F1329 (Takamine)			8	II
Gumase HP150 mix 10 (R and H)			22	I
Snail, *Helix* (Ind. Biol. Franc.)			1.8	V

NOTE: Commercial sources: Takamine Div., Miles Chem. Co., Elkhart, Indiana; Rohm and Haas Co., Philadelphia, Pa.; Industrie Biologie Francaise, Gennevilliers (Seine). Snail enzyme is a solution; units/mg solution.
*The yield shown is the highest we have obtained over a series of experiments in which variations were made in the medium and in conditions of growth.
†"Type" refers to the glycosidases present with the β-mannanase, as shown in Fig. 4.

Results

1. Search for β-1 → 4 Mannanase

Over 290 cultures were tested for their ability to produce β-mannanase in shake flasks on a basal medium to which mannan had been added. Most of these produced at least trace amounts of the desired enzyme. The most active organisms (Table I) are *Penicillium wortmanni*, *Aspergillus giganteus*, and *Paecilomyces varioti*. Sixty commercial enzyme preparations, mostly carbohydrases, were tested for β-mannanase activity. The best of these are shown in Table I. That very few have high activity is understandable since β-mannanase is adaptive. Thus, commercial enzyme preparations derived from *B. subtilis* are generally devoid of mannanase, yet this organism, grown on mannan, is one of our better sources of enzyme.

Trichoderma viride and *Chaetomium globosum* were found by Lyr (18) to be very active producers of mannanase. The yields which we obtained for the same fungi are less than 10 of our units per milliliter. We believe that the organisms (Table I) found by our more extensive screening procedure are considerably more active than those previously reported.

2. β-1 → 4 Mannanases as Inducible Enzymes

All of the active microorganisms produce small amounts of β-mannanase even when grown on simple sugars or on starch. When grown on ivory nut meal, ivory nut mannan, or carob gum, these organisms were greatly stimulated in their output of enzyme, the increase being 10- to more than 100-fold (Table II).

Lyr (18) observed that some Basidiomycetes produce larger amounts of β-mannanase when grown on cellulose than when grown on mannan. We, too, have observed instances of this behavior with other fungi. For instance, Basidiomycete sp. QM 806 produced 11 units (on cellulose) vs. 3 units (on mannan) and *Sporotrichum pruinosum* QM 826 produced 21 units (on cellulose) vs. 3 units (on mannan). However, this was not the usual case, and these mannanase yields are low compared to those of our better organisms (Table I). It is

TABLE II

Effect of growth substrate on production of β-1 → 4 mannanase

Organisms	QM No.	β-1 → 4 mannanase, units/ml (max.)				
		Mannose	Glucose	Starch	Mannan	Carob
Aspergillus fumigatus	45h	0.6	1.0	0.8	18	32*
A. giganteus	7974	0.0	0.0	0.0	12	3*
A. luchuensis	873	1.7	0.9	—	18	30
A. nidulans v. *echinulatus*	1909	0.0	0.0	—	10	3
Bacillus subtilis	B1564	1.5	1.5	—	125	60
Cladosarum olivaceum	2000	1.8	0.8	0.6	9	7*
Gelatinosporium sp.	684	1.5	1.3	—	18	19
Fusicoccum sp.	2045	1.2	0.8	1.0	5	72
Paecilomyces varioti	3408	3.0	2.8	2.8	60	265*
Penicillium funiculosum	474	0.4	0.4	0.4	49	8*
P. ochro-chloron	477	0.6	0.0	—	72	18
P. verruculosum	7999	0.3	0.0	0.2	25	142*
P. wortmanni	1859	2.1	0.0	—	530	400

*In these, the carob gum was partially hydrolyzed (enzymes) before addition to the medium.

interesting that, in our experience, cellulose also induces xylanase (in QM 806, 826, and others). It is unlikely that this induction results from a contaminating mannan or xylan in the cellulose since the effect is observed with cellulose of diverse origins. This inducing effect is peculiar to cellulose and is specific for certain organisms.

3. β-1 → 4 Mannanase Preparations

(a) Properties

Most β-mannanases, like other polysaccharases, are rather stable enzymes. Except for a few, they can be recovered from cold culture filtrates by acetone (or alcohol) precipitation. For the exceptions, those of *Aspergillus giganteus*, *Trichurus spiralis*, and *Gelatinosporium* sp., the activity can be recovered in good yields by evaporation of dialyzed culture filtrates under reduced pressure in the presence of celite, with a final drying from methanol.

The pH vs. activity curves of several preparations showed activity from pH 2.0 to 8.0. Most followed the same pattern between pH 6.0 and 8.0. Below pH 4.5 the enzymes may be divided into two groups: those like *A. giganteus* (Fig. 2) which were relatively inactive at pH 3.5 (snail enzyme, *B. subtilis*), and those like *P. verruculosum* which had relatively high activity even at pH 3.0 (*P. funiculosum*, commercial enzymes, *P. ochro-chloron*, *P. wortmanni*, *A. luchuensis*, *Paecilomyces varioti*, and *Fusicoccum* sp.). The shape of the latter curves may indicate the presence of more than one mannanase component, one of which is most active at low pH.

The temperature vs. activity curves (Fig. 2) also indicated a high degree of stability. In the 1-hour assay at pH 4.5, all enzyme preparations showed maximum activity at 60 °C or above (70 °C was the highest tested).

The effect of substrate concentration on rate of hydrolysis is difficult to assess with substrates containing more than one linkage type and involving the action of more than one enzyme. In examining this relationship for β-mannanases, partially degraded products of carob gum were used to avoid the

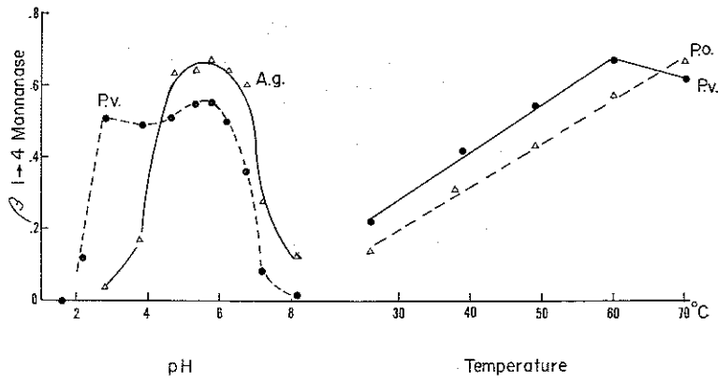

FIG. 2. Activity of β-1 → 4 mannanase. Effect of pH (50 °C, 30 minutes). Effect of temperature (pH 4.5, 60 minutes). Enzymes of *Penicillium verruculosum* 7999 (P.v.), *Aspergillus giganteus* 7974 (A.g.), *Penicillium ochro-chloron* 477 (P.o.). β-mannanase activities are shown as glucose mg per milliliter.

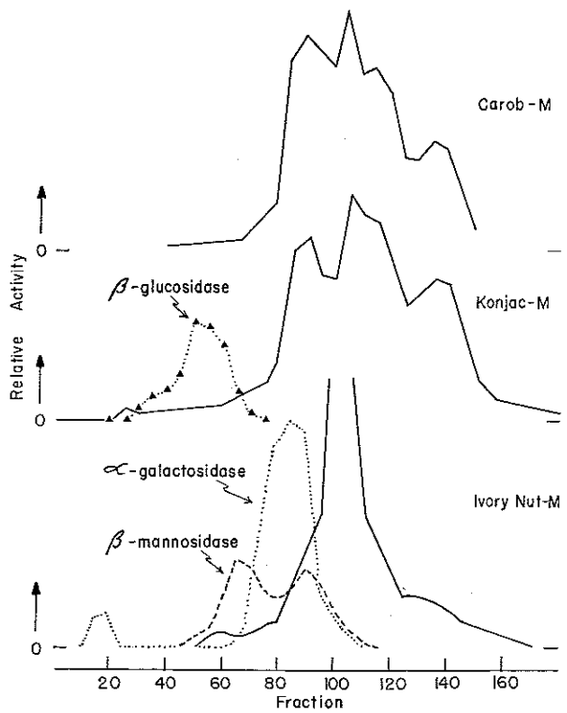

FIG. 3. Separation of enzyme components of *Penicillium funiculosum* on DEAE-Sephadex. NaCl gradient from 0 to ca. $M/5$ in $M/50$ potassium phosphate buffer, pH 6.9, 5 ml fractions. Enzyme activities are in arbitrary units based on hydrolysis of salicin, 1 hour (β-glucosidase); *p*-nitrophenyl β-galactoside, 15 minutes (α-galactosidase); methyl β-mannoside, 24 hours (β-mannosidase); carob mannan, 30 minutes; Konjac and ivory nut mannan, 1 hour (β-mannanase: M), temp. 50 °C; pH 4.5.

necessity of working with highly viscous solutions. The effect of substrate concentration on initial rate was relatively slight. Over the range 0.5–16 mg substrate per milliliter, the maximum rate was less than twice that found at the lowest concentration tested.

(b) Separation of Components of Mannanase Systems

Several crude preparations have been chromatographed on DEAE-Sephadex columns in an attempt to obtain purified enzyme. With *P. funiculosum* (Fig. 3), there were at least three peaks for activity (reducing groups) on galactomannan (carob) and glucomannan (Konjac), but only a single major peak for activity on ivory nut mannan. (Assays by the carob viscosity method show a single peak similar to the latter.) The peak activity (carob) in tube 90 increased relative to that in tube 105 with time of incubation, influenced apparently by the presence of α-galactosidase and β-mannosidase. The No. 135 component was relatively inactive against the insoluble ivory nut mannan. In this experiment, β-glucosidase was well separated from β-mannanases

permitting study of glucomannan hydrolysis with the individual enzymes. Except for the No. 135 mannanase, separation of α-galactosidase and of β-mannosidase (two components) from mannanase was not good.

In other experiments (not shown), a good separation of β-glucosidase from β-mannanase was obtained for *A. luchuensis*, but again the α-galactosidase overlapped the β-mannanase. In *A. giganteus*, two sharp mannanase peaks (95, 125) of equal size were obtained when measurement was by viscosity change of carob gum. These coincided with two less-well-resolved components as determined by reducing sugar production from the same substrate. The second major peak (125) was contaminated with α-galactosidase; the first was free of α-galactosidase and of β-mannosidase. With hemicellulase (Table I), an excellent separation was obtained of β-mannanase (one peak at 88) from β-mannosidase (one peak at 40). There was no detectable α-galactosidase in this preparation.

The results show that β-mannanase may exist as a single component, or as several components, and that the chromatographic patterns vary depending upon the method of measurement used and upon the presence or absence of contaminating glycosidases. To date, each of the enzymes, except α-galactosidase, has been obtained free of the others.

(c) Specificity of β-Mannanase

The mannanases under investigation hydrolyze mannans having β-linkages. They did not hydrolyze the α-mannans of yeast or phosphomannans (also α-linked). No β-mannans of linkage other than 1-4 have been available for testing. By analogy with other polysaccharases, we assume that the β-mannanases studied have a requirement for the β-1,4 linkage between mannose units and that β-mannans linked otherwise (if such exist) will not be attacked. (For effect of side groups on hydrolysis of galactomannan, see Discussion.)

The effect of chain length on β-mannanase activity has not been specifically investigated. The persistence of trimer and dimer in hydrolysates (by preparations lacking β-mannosidase) indicates that the chain length must be greater than 3 units for any appreciable activity to occur. A pentamer was hydrolyzed (by *A. giganteus* mannanase) to dimer and trimer. It is probable that the tetramer is hydrolyzed at a somewhat lower rate. (A search for α-mannanase is underway, but to date it has been unsuccessful.)

4. Glycosidases Associated with β-Mannanases

(*a*) At least three enzymes are involved in the hydrolysis of galactomannans: β-1 $\rightarrow$ 4 mannanase, β-mannosidase, and α-galactosidase. The preparations produced from microorganisms varied in the relative amounts of each of these components. Unfortunately, our substrates for measuring glycosidase activities (α-CH_3 galactoside and β-CH_3 mannoside), are refractory materials, so that the levels observed for these enzymes are quite low (usually less than 0.1 units/mg). However, the hydrolysis products of the galactomannans indicated that the glycosidase levels are often sufficient to result in the production of appreciable amounts of the simple sugars.

Based on the chromatographic patterns (Fig. 4), the enzyme complexes of different organisms can be put into five categories depending upon the glyco-

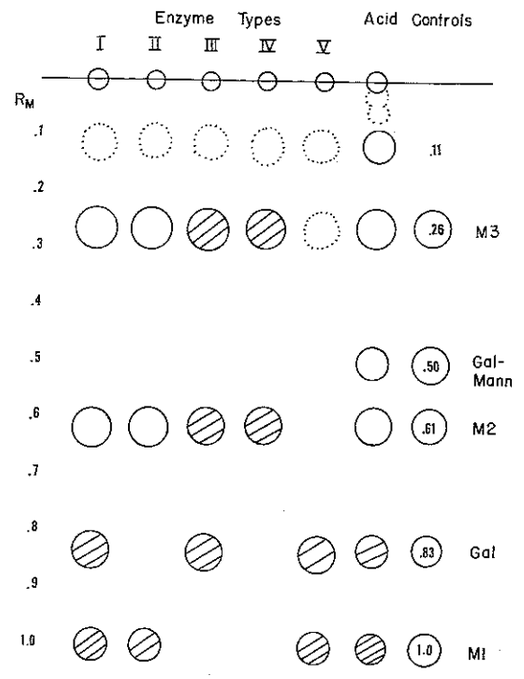

FIG. 4. Hydrolysis products of carob gum (diagrammatic). Enzyme type:
(I) mannanase and β-mannosidase + α-galactosidase: *P. wortmanni*;
(II) mannanase + β-mannosidase (no galactosidase): *Paecilomyces varioti*;
(III) mannanase (no mannosidase), + galactosidase: *A. giganteus*;
(IV) mannanase (no mannosidase, no galactosidase): *Bacillus subtilis*;
(V) mannanase weak relative to both glycosidases: snail (*Helix*).
Hydrolysis conditions: 0.33 *N* H_2SO_4 100°, 4 hours. Solvent: isopropanol:acetic acid:water::54:8:18. R_M values are based on mannose. Authentic markers are M2 = β-1 → 4 dimannose; M3 = β-1 → 4 trimannose; Gal-Man = 6-O-α-galactopyranosyl D-mannose.
Cross hatching is an indication of intensity of the spots. Dotted circles represent weak components, visible only under ultraviolet light. In all tests, only a limited hydrolysis has occurred.

sidases present (Table I). Obviously, these categories are not absolute, and in the presence of a low enzyme concentration a very long incubation period will yield sufficient monosaccharide to be visible on the chromatogram. The classification is useful, however, for indicating enzyme preparations suitable for different purposes. One may desire either complete hydrolysis (types I, V), or an accumulation of intermediates (II, III, IV).

The production of dimer, trimer, and other oligosaccharides indicates that all of the β-1,4-mannanases examined are endoenzymes, acting in a random fashion. Occasionally, mannose and galactose are the dominant products (from galactomannan), as in type V of our classification. That this is *not* an example of endwise (exomannanase) action has been demonstrated with the snail enzyme. A purified fraction, freed of most of the mannosidase on DEAE-Sephadex, yields M2 and M3 in appreciable amounts. In hydrolysis by the

TABLE III
Specificity of β-mannosidases

Enzyme	Hydrolysis, %*				
	M2	M2R	M3	M3R	β-CH_3-M
1. *Penicillium funiculosum* QM 474	26	3	37	39	10
2. *P. ochro-chloron* QM 477	26	2	34	33	5
3. Snail (*Helix pomatia*)	46	3	59	40	5

*Substrate 0.0068 *M*, pH 4.5, 1 hour 50°. Enzymes 1 and 2 at 0.5 mg/ml reaction mixture, 3 at 1/200 vial/m of commercial enzyme (Industrie Biologique Francaise, 1 ml/vial). These have not been freed of β-mannanases. M2 = mannobiose, M2R = reduced mannobiose, M3 = mannotriose, M3R = reduced mannotriose. β-CH_3-M = β-CH_3-mannoside.

whole snail enzyme, these oligosaccharides were rapidly hydrolyzed to mannose.

(*b*) *β-Mannosidase* is the enzyme responsible for the hydrolysis of the mannobiose and mannotriose (produced by mannanase) to mannose. Its absence in mannanase preparations resulted in an accumulation of the oligosaccharides during mannan hydrolysis (Fig. 4, III, IV). Mannotriose was hydrolyzed somewhat faster than mannobiose (Table III). Cellobiose and cellotriose were hydrolyzed at about the same rate by β-glucosidases we have studied, except for almond emulsin which was more active on the dimer (unpublished observations). Reduced mannobiose was highly resistant to mannosidase. This resistance is common to all reduced dimers with which we have worked (cellobiitol, lactitol, maltitol) or seen reported, a phenomenon for which we have no explanation. Reduced trimers, on the other hand, were as readily hydrolyzed as the trimers themselves and are good substrates for the glycosidase inasmuch as they are non-reducing and yield reducing products on hydrolysis. β-CH_3-Mannoside, like other β-CH_3-glycosides, was the most resistant of all compounds tested, except the reduced dimer.

The action of β-mannosidases on reduced mannotriose was not inhibited by γ-mannonolactone (gift of Dr. Isbell) at a lactone:substrate ratio of 0.5. Levvy *et al.* (17) have found that the Δ-lactone is over 500 times as active as the γ-lactone. Our tests with a sample of the Δ-lactone (gift of Dr. Conchie) confirmed their findings, the ratio L/S for 50% inhibition being 0.0005 for snail enzyme, 0.0025 for that of *P. funiculosum*, and 0.009 for that of *P. ochro-chloron*. The Δ-mannonolactone is a very active and specific inhibitor of the β-mannosidases. It did not affect the action of the α-galactosidases or of the β-mannanases in our tests on carob hydrolysis.

(*c*) *α-Galactosidase* is required for the complete hydrolysis of galactomannans and is present in some but not in all of the β-mannanase preparations. An attempt was made to obtain a preparation of this enzyme free of the mannanase. Over 40 organisms were grown on galactosides (melibiose, melibiose octaacetate, raffinose) and on sucrose monopalmitate, and tested for activity against α-CH_3-galactoside. *Aspergillus fumigatus*, grown on sucrose monopalmitate, gave the most α-galactosidase and the enzyme was liberated into the medium. *A. luchuensis* was the next best organism, but the enzyme was retained in the mycelium and had to be liberated by grinding. In both preparations, β-mannanase levels were very low (< 0.3 units/ml). Unfortunately,

although these α-galactosidases are active on α-CH_3-galactoside, stachyose, and melibiose, they have little or no ability to remove galactose from carob or guar. Courtois seems to have been similarly disappointed (7, 12). The specificities of his microbial and animal α-galactosidases (oyster, rabbit kidney) resemble those of *A. fumigatus*.

The α-galactosidases found in mannanase preparations have greater specificity for the galactose of carob and of guar than for that of the galactosides (above). In the absence of a quantitative assay based on guar, this conclusion rests on chromatograms of the hydrolysis products of the various galactosides. However, even the galactosidases present in mannanase preparations may vary in specificity or may be mixtures of types. A comparison of their activities on α-CH_3-galactoside vs. reduced manninotriose (Gal-1-α-6-Gal-1-α-6-sorbitol) shows equal activities for some preparations (e.g., *P. funiculosum* 0.58 vs. 0.57 mg reducing sugar/ml 2 hours) and different activities for others (*P. ochro-chloron* 0.29 vs. 0.76).

Attempts to inhibit the α-galactosidases by means of γ-galactonolactone have been unsuccessful. At lactone:substrate ratios up to one, there was no appreciable decrease in the amount of galactose produced from guar or carob.

(*d*) *β-Glucosidase* is involved in the complete hydrolysis of the glucomannans. It is present in most of the mannanase preparations we have examined (Table I), but absent, or nearly so, in those preparations lacking β-mannosidase, for instance, those of *A. giganteus*, *Trichurus spiralis*, and *Bacillus subtilis*. The latter can thus be used to obtain oligosaccharide products containing glucose. These products in turn can be subjected subsequently to β-glucosidase action. A β-glucosidase, free of mannanase, has been obtained from *A. luchuensis* by separation on DEAE-Sephadex. Glucose units on the non-reducing end of short oligosaccharide chains should be removable by this enzyme.

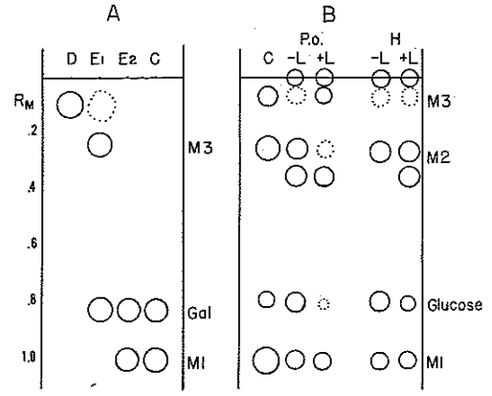

FIG. 5. A. Analysis of R_M 0.10 component of carob digest. D = component + buffer. E_1 = component + *A. giganteus* mannanase preparation. E_2 = component + *P. ochro-chloron* mannanase preparation. C = galactose + mannose (M1) control. Develop in isopropanol:acetic:water::54:8:18.

B. Effect of β-glucosidase on Konjac mannan digestion. Hydrolysis 3 hours, 50° + Δ-gluconolactone (L). Gluconolactone:Konjac mannan::1:5. C = M1, M2 (dimannose), M3 (trimannose), and glucose. Developed in EtOAc:HOAc:H_2O::9:2:2. Enzymes: P.o. = *P. ochro-chloron*. H = hemicellulase (R. and H.).

TABLE IV

Products of enzyme and acid hydrolysis of mannans

Hydrolysis by:	Products M1	M2	M3	M4+
Enzyme				
Penicillium wortmanni, 16 hr 50°	19	66	16	0
Penicillium wortmanni, 16 hr 50°	25	68	7	0
Aspergillus giganteus, 16 hr 50°	1	41	45	13
Penicillium funiculosum, 16 hr 50°	17	68	15	—
Guar seed* (Whistler, 29), 72 hr 35°	83	8	9	—
Acid				
(0.4 *N* $H_2SO)_4$, 2 hr 100°	56	23	11	9

NOTE: Hydrolysis of ivory nut mannan, except for Whistler's data (*) where guar gum was the substrate. Values based on carbon column fractionation and indicate the relative amounts (by weight) of the mannose oligosaccharides where M1 = mannose; M2 = mannobiose, etc.

Δ-Gluconolactone was found to be an excellent inhibitor of β-glucosidase activity. Its presence in glucomannan hydrolysis mixtures decreases the amount of glucose produced, and increases certain of the intermediates (Fig. 5B), those susceptible to hydrolysis by β-glucosidase.

5. Hydrolysis Products of the Various Mannans

Ivory nut mannan was hydrolyzed to the same products as galactomannan (Fig. 4), except that there is no galactose. Most microbial enzyme preparations yielded M2 as the major product (Table IV), though this depends upon the incubation period used. In the absence of β-mannosidase (*A. giganteus*, Table IV), M3 and longer fragments also accumulated. The guar seed enzyme preparations (Table IV) are obviously richer in glycosidases than in mannanases since relatively little M2 and M3 were found. Acid hydrolysis also yielded mostly monosaccharides.

Two galactomannans differing in galactose content (Fig. 1) were investigated. The mannanase preparation of *Penicillium wortmanni* (which contains α-galactosidase and β-mannosidase) extensively hydrolyzed (Fig. 6) carob and guar gums. (Carob does contain a resistant fraction, about 7%, which precipitated from the reaction mixture soon after hydrolysis begins.) The mannanases, essentially free of both glycosidases, as in *Trichurus spiralis* (Fig. 6) and *Bacillus subtilis* (not shown), achieved an initial rapid hydrolysis of both guar (9%) and carob (49%). Further hydrolysis was very slow. The products of complete hydrolysis (*P. wortmanni*, at 6 days) were galactose and mannose. From the partial hydrolysis by *T. spiralis*, mannobiose was the major "small" product from carob, and no small fragment (less than 5 unit length) was obtained from guar (chromatographic data). Most of the products of *T. spiralis* action must be of appreciable size, and we believe that this enzyme acts only on portions of the original polymer that are somewhat less substituted (or branched) than the rest. (Hui and Neukom (14) subjected these two galactomannans to the action of *α-galactosidase* of coffee beans. They claim 20% removal of galactose from guar, and 70% from carob. Since

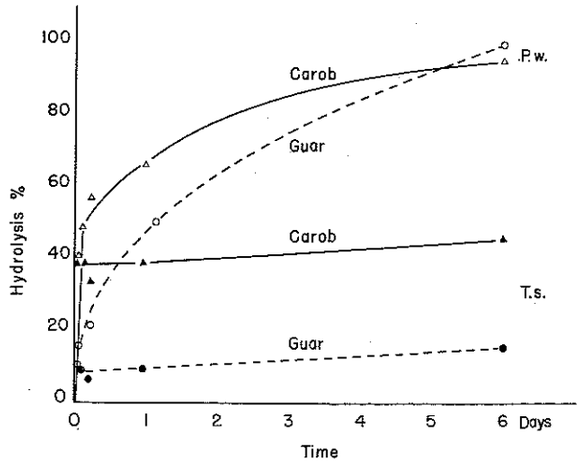

FIG. 6. Hydrolysis of galactomannans by enzymes. β-Mannanase preparation containing both β-mannosidase and α-galactosidase (P.w. = *Penicillium wortmanni* at 600, β-mannanase units/ml (○, △). β-Mannanase preparation free of β-mannosidase and of α-galactosidase (T.s. = *Trichurus spiralis* at 100 units/ml ●, ▲). Conditions: substrate 2 mg/ml; *M*/20 citrate, pH 4.5; temp. 50°.

galactose was not specifically determined, we question their interpretation and suggest that they were really measuring activity of β-mannanase present in their enzyme preparation.)

To have a better look at the hydrolysis products of carob gum, a 10 g sample was subjected to the action of *T. spiralis* enzyme preparation (ca. 4000 β-mannanase units) for 2 days at 50°. An insoluble residue of low galactose content remained (7%). Fractionation of the soluble products with alcohol gave 1% of product precipitated with 1 volume ethanol, 48% with 3 volume ethanol (fraction C3), and 33% was recovered from the alcohol solution (fraction C4). Mannobiose and somewhat less mannotriose were the major small components of C3 and C4, but there were traces of two slower moving components on the chromatograms. One, the R_M 0.10 component, was separated by paper chromatography and by chromatography on stearic acid treated carbon. It amounts to about 6% of the C_3 fraction (= 3% of initial carob). Complete hydrolysis of R_M 0.10 by *P. ochro-chloron* enzyme gave mannose and galactose (Fig. 5A). Hydrolysis by the *A. giganteus* enzyme preparation gave mannotriose and galactose. This R_M 0.10 component appears to be the smallest mixed oligosaccharide produced by *T. spiralis* enzyme acting on carob gum. Although the position of the galactose has not been determined, we believe it to be on the middle mannose unit. (6-O-α-Galactosyl mannose has never been observed as a product of enzyme digestion, though it is common enough in the acid hydrolysates.)

Konjac glucomannan was rapidly hydrolyzed by all of the *B*-mannanase preparations as observed by reduction in viscosity and increase in reducing value with DNS. To obtain an improved substrate, that is, one of low viscosity, the glucomannan was heated to 100 °C in 0.1 *N* HCl for 15 minutes. There were no fragments produced small enough to be detected on paper

chromatograms. All of the β-mannanases that contain β-glucosidase (this includes the four commercial preparations, Table I) hydrolyzed this substrate completely to glucose and mannose). Partial hydrolysis by the same enzymes yielded three dominant intermediates (Fig. 5B).

The *trimer*, though it moved at about the rate of mannotriose, must have two mannoses and one glucose (Fig. 1). β-Glucosidases acted on this to give glucose and mannobiose, thus indicating the probable arrangement G—M—M.* (The possibility of other arrangements has not been excluded.)

The *dimers* are at least two in number. The faster moving one (in two different solvents) behaved like an authentic sample of 4-O-β-glucosyl mannose. β-Glucosidase hydrolyzed it to glucose plus mannose. The slower-moving spot coincides with both 4-O-β-mannosyl glucose and 4-O-β-mannosyl mannose (in the two solvents). On enzymic hydrolysis, it yields primarily mannose. (A trace of glucose may be the result of contamination of the sample with 4-O-β-glucosyl mannose, from which it had been separated on paper chromatograms.) The second dimer is, then, mannobiose. The 4-O-β-mannosyl glucose is either absent or present only in trace amounts.

The β-mannanases that lack β-glucosidase (*A. giganteus*, *B. subtilis*, *T. spiralis*) act on Konjac glucomannan in a different way. Even after extended incubation periods, only a small percentage of the products were small enough to move on paper chromatograms. (Those that did move may result from heterogeneity in the initial material.) Most of the hydrolysate consisted of products longer than 5 sugar units. Their resistance to further decomposition by mannanase may indicate a branched structure. Purified β-glucosidase removed glucose from these, without sufficiently shortening the chains to permit their movement on paper chromatograms.

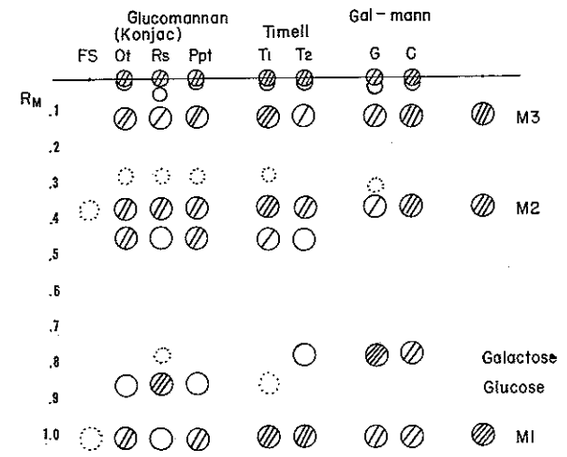

FIG. 7. Acid hydrolysis products of mannans. Acid hydrolysis of 20 mg sample in 1 ml 0.33 *N* H_2SO_4, 100°, 2 hours. Samples subjected to hydrolysis were Konjac mannan: FS = precipitated with Fehling's solution; Ot = sample from Otsuki; Rs = residue after enzyme (*A. giganteus*) digestion; Ppt = partially hydrolyzed, and precipitated with alcohol (1 v., 3 v.). Timell galactoglucomannans: T_1 = 0.1:1:3; T_2 = 1:1:3 (proportion of sugars). Galactomannans: G = guar; C = carob.

Acid hydrolysis (partial) of the glucomannans also yielded two dimers separable in EtOAc–HOAc–water but not separable in isopropyl–acetic–water (Fig. 7). The faster spot is glucosyl mannose; the slower, dimannose and (or) mannosyl glucose. The galactoglucomannans of Timell yielded less glucosyl mannose than did Konjac mannan. The trimer which moved at the same rate as mannotriose in the two solvents is probably composed of two mannose and one glucose units. Glucose and mannose were the monomeric products. The acid hydrolysis products indicated that our partially degraded Konjac mannan (Ppt) has essentially the same composition as a sample prepared by Otsuki. The residue which resisted enzyme hydrolysis was relatively richer in glucose and poorer in mannose; and the product prepared by precipitation with Fehling's solution was highly resistant to acid hydrolysis (Fig. 7).

Acid hydrolysates of guar and carob contained chiefly the one dimer, mannobiose. In the guar hydrolysate, however, a trace of a slower component was evident. This moved at the rate of 6-O-α-galactosyl mannose, earlier isolated and described by Whistler and Durso (28). Mannotriose, galactose, and glucose were the other products.

Discussion

We have found enzyme mixtures which hydrolyze the various mannans either completely, or partially. The preparations suitable for *complete* hydrolysis to monomeric units contain not only β-mannanase, but also the glycosidases: β-mannosidase, β-glucosidase, and α-galactosidase. Usually a small part of each mannan resists digestion, and precipitates soon after the solution loses its viscous nature. The enzyme preparations that give only *partial* hydrolysis lack the glycosidases, and permit the accumulation of the oligosaccharide fragments. Where the initial mannan is simple (e.g., ivory nut), the oligosaccharides are predominantly mannobiose and mannotriose. Where branching is present (e.g., carob gum, etc.), the oligosaccharides contain branched products, and the amount of unbranched product decreases rapidly as the number of branches increases. Where the mannan backbone is interspersed with glucose units and branches (e.g., Konjac mannan), most of the oligosaccharide fragments are quite large, and appear to have glucose as a non-reducing terminal unit. These oligosaccharides can be further partially or completely degraded by addition of the glycosidases (and by β-mannanase if the resulting oligosaccharide is larger than 3 units). It is only by the secondary action of these glycosidases that debranching takes place, giving rise to the hetero-dimers (e.g., 4-O-β-glucosyl mannose, etc.).

Substitution and its Effect on β-Mannanase Activity

An endoxylanase requires for its activity at least two adjacent *un*substituted units in the arabinoxylan chain (Perlin *et al.* (22)). It is likely that this requirement can be applied to other endoenzymes. The rapid initial hydrolysis of galacto- and gluco-mannans (by preparations devoid of glycosidases capable of removing side groups) probably indicates activity of the β-mannanase in less substituted regions of the mannan molecule. Guar, partially hydrolyzed by *T. spiralis* enzymes, yields a product that has a ratio of mannose:galactose::2:1, with a galactose unit on every other mannose unit of the chain.

The β-mannanases are unable to hydrolyze the following structure.

$$-M-M-M-M-M-$$
$$\quad\;\;|\qquad\;\;|$$
$$\quad Gal \quad Gal$$

A single unsubstituted mannose unit between branches does not allow sufficient room for the approach of the enzyme to the site to be hydrolyzed. As a result, a degree of substitution (D.S.) of 0.5 (one substituent per two units of the chain) is sufficient to permit a polymeric structure to resist hydrolysis by endoenzymes. This is much below the value (D.S. 1.0) that we previously assigned based on substituted celluloses and their resistance to cellulases. We believe now that the inability to substitute cellulose uniformly led us to the higher value. Undoubtedly, pairs of unsubstituted glucoses exist even in celluloses of high D.S. For resistance at D.S. 0.5, it is necessary that substitution be uniformly on every other sugar unit of the linear polymer.

The hydrolysis products of arabino xylan (by the endoxylanase) contain the branch at an interior unit, never on the end unit of the oligosaccharide (Perlin (22)). If Perlin's conclusions are applicable to this system, the branched oligosaccharides resulting from enzyme hydrolysis of galactomannan should be branched at an interior mannose unit, the simplest case being

$$M-M-M$$
$$\quad\;|$$
$$\quad Gal$$

which we tentatively assign as the structure of the R_M 0.10 spot from carob.

Removal of Side Groups from Branched Polysaccharides

The α-galactosidases present in some of our mannanase preparations hydrolyze off galactose units from guar and carob gums. They are definitely active on oligosaccharides, as seen when the R_M 0.10 component was hydrolyzed to M–M–M and galactose. As we have had no α-galactosidase free of mannanase, we are unable to say whether these enzymes can act on very long chains. Unlike the endomannanases, they can operate in close quarters, such as on guar products where galactose is found on every other mannose unit, as well as on the more open structure of the carob oligosaccharides.

It is interesting that this α-galactosidase is relatively inactive on some common α-galactosides, for instance, α-CH_3 galactoside, melibiose, and stachyose (see also ref. 12). Another α-galactosidase (*A. fumigatus*), prepared specifically for the present work, turned out to be a disappointment because it behaved in the opposite manner, that is, it was active on α-CH_3 galactoside and inactive on guar fragments. There is thus a neat problem in specificity and a need for a good substrate for assaying that α-galactosidase of interest in the present context.

β-Glucosidase actively removes glucose from oligosaccharide fragments of Konjac glucomannan. A purified β-glucosidase of another source (*A. luchuensis*) can do this also, but it appears to have no effect on the initial glucomannan. Since this enzyme acts from the non-reducing end, the oligosaccharides must be of the type G–M–M*, G–M–M–G–M–M*, etc. The fact that relatively little of the trimer is produced in the absence of glycosidases leads us to believe that the oligosaccharide contains a branch (either glucose

or mannose) which interferes with the action of β-mannanase. The presence of a glucose unit at the non-reducing end of these fragments is in agreement with our theory (23) on the action of endopolysaccharases which says that, in this instance, the β-mannanases have an affinity for mannobiose cleaving at the adjacent linkage, yielding oligosaccharides having the mannose dimer at the reducing end of the fragment. Further analysis of these products is required.

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